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# IL-7 unveils pathogen-specific T cells by enhancing antigen-recall responses.

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**Running title:** IL-7 sustains in vivo-primed T cells

## Summary

IL-7 supports host immunity in lymphopenic and immunosuppressed patients and expand tumor-reactive T cells for adoptive immunotherapy. Here, we report that IL-7 also enables

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the accumulation of clinically-relevant pathogen-reactive T cells from TB, CMV, and *Candida albicans* infected individuals.

## Abstract

**BACKGROUND:** IL-7 promotes the generation, expansion and survival of memory T cells. Previous mouse and human studies showed that IL-7 can support immune cell reconstitution in lymphopenic conditions, expand tumor-reactive T cells for adoptive immunotherapy and enhance effector cytokine expression by autoreactive T cells. Whether pathogen-reactive T cells also benefit from IL-7 exposure remains unknown. **METHODS:** Here we investigated this issue in cultures of peripheral blood mononuclear cells (PBMCs) derived from patients infected with various endemic pathogens. After short-term exposure to IL-7, we measured PBMC responses to antigens (Ag) derived from pathogens, such as *Mycobacterium tuberculosis* (MTB), *Candida albicans* (Ca) and Cytomegalovirus (CMV), and to the superantigen *Staphylococcus aureus* enterotoxin B (SEB). **RESULTS:** We found that IL-7 favoured the expansion and, in some instances, the uncovering of pathogen-reactive CD4<sup>+</sup> T cells, by promoting pathogen-specific IFN $\gamma$ , IL-2 and TNF recall responses. **CONCLUSIONS:** Our findings indicate that IL-7 unveils and supports re-activation of pathogen-specific T cells with possible diagnostic, prognostic and therapeutic significance, of clinical value especially in conditions of pathogen persistence and chronic infection.

**Keywords:** IL-7; pathogen-specific responses; memory T cells; polyfunctional T cells; TB infection; candida infection; CMV infection; SEB responses; in vivo priming; T cell expansion.

## Footnotes

**Conflict of interest:** SC and AM declare to be inventors in a patent application for the use of common- $\gamma$  chain cytokines for the visualization, isolation and genetic modification of memory T lymphocytes (US 2010.0035282A1). The other authors have no conflict of interest.

**Presentation in previous meetings:** the data contained in this manuscript has never been presented in full in any regional, national or international meetings. However, early data was partly included in a poster presentation at the “4<sup>th</sup> European Congress of Immunology”, 6-9 September 2015, Vienna, Austria.

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**Abbreviations used in the text:** IL-: interleukin; Ag: antigen; MTB: *Mycobacterium tuberculosis*; Ca: *Candida albicans*; CMV: Cytomegalovirus; SEB: *Staphylococcus aureus* enterotoxin B; TNF: tumor necrosis factor; IFN: interferon; IL-7R: interleukin-7 receptor; PBMCs: peripheral blood mononuclear cells; TCM: central memory T cells; TEM: effector memory T cells; TSCM: stem-cell memory T cells; TCR: T cell receptor; Th-: T helper cell; ACT: adoptive T cell therapy; HIV: human immunodeficiency virus; SIV: simian

immunodeficiency virus; TB: tuberculosis; TST: tuberculin skin test; MTP: *Mycobacterium tuberculosis*-specific peptides; BCG: *Bacillus Calmette Guérin*; APCs: antigen-presenting cells; CMV<sup>-</sup>: Cytomegalovirus-seronegative; CMV<sup>+</sup>: Cytomegalovirus-seropositive; PBS: phosphate-buffered saline; AICD: activation-induced cell death; FCS: fetal calf serum; RPMI: Roswell Park Memorial Institute medium; CFSE: 5-(and-6)-carboxyfluorescein diacetate succinimide ester; CSA: cyclosporine A; *ELISPOT*: enzyme-linked immuno-spot assay; *SFC*: spot forming cell; *ICS*: intracellular cytokine staining; *mAb*: monoclonal antibody.

## Introduction

IL-7[1] is a pleiotropic cytokine[2] regulating lymphopoiesis and T cell homeostasis[3, 4]. It binds to a heterodimeric receptor formed by an  $\alpha$ -chain (CD127[3]), which is private to the IL-7 receptor (IL-7R) and the common- $\gamma$ -chain cytokine-receptor (CD132[5, 6]). In humans, mutations negatively affecting the levels of CD127 have been correlated with severe immunodeficiency[7]. Conversely, increased serum levels of IL-7 and/or dysregulated activation of CD127 are reported in patients with autoimmune conditions, including multiple sclerosis[8, 9], rheumatoid arthritis[10], type-I diabetes[11], inflammatory bowel disease[12] and psoriasis[13].

Upon antigen encounter, IL-7 sustains the generation of memory T lymphocytes in vitro[14] and in vivo[14-16] and favors the transition of effector to central memory cells[17, 18] while driving their proliferation[19]. Via the JAK3/STAT5 pathway and the upregulation of anti-apoptotic factors (e.g., Bcl-2)[14, 20], IL-7 promotes the long-term survival of naïve and memory-phenotype cells. Recombinant IL-7 elicits a marked increase of central memory (T<sub>CM</sub>) and effector memory T cells (T<sub>EM</sub>) when administered to aged non-human primates[21], and lymphopenic patients and macaques infected with HIV or SIV respectively[22-24]. Similar effects are reported in patients undergoing stem cell transplantation[25, 26], where IL-7 levels correlate with the generation of stem-cell memory T cells (T<sub>SCM</sub>)[26]. In cancer patients, IL-7 preferentially increases naïve, but not regulatory T cell numbers[27], maintaining T cell receptor (TCR) repertoire diversity[28]. Hence, the

immunotherapeutic use of IL-7 is increasingly proposed to favor immune-cell reconstitution and function after lympho-depleting chemotherapy or in the elderly. In the setting of adoptive T cell therapy (ACT), IL-7 has been used in primary cultures to engineer and expand tumor-reactive T cells[29, 30]. We previously found that IL-7 selectively expands tumor-reactive CD4 T cells capable of promoting tumor protection in ACT[29]. Whether similar results could be extendible to pathogen-specific T cells remains unknown. We therefore sought to investigate the expansion of pathogen-reactive CD4 T cells in individuals affected by recurrent or persistent/chronic bacterial (*Mycobacterium tuberculosis*, MTB and *Staphylococcus aureus*), fungal (*Candida albicans*, Ca) or viral (Cytomegalovirus, CMV) infections. We report that, in all cases, IL-7 enriched pathogen-specific CD4 T cells, enabling their detection and sensitizing them to antigen-specific recall responses. Further, IL-7 rescued chronically activated pathogen-specific effectors enhancing their Ag-recall responses. We believe that these data open new avenues for diagnostic, prognostic and therapeutic applications.

## Materials and Methods

### Classification of TB patients.

HIV-seronegative patients with active TB (clinic and culture confirmed) were recruited at the Clinic of Infectious Diseases, San Raffaele Hospital (Milan, Italy). They underwent TST administered by the Mantoux method with 0.1 ml (5 tuberculin units) of Biocinetest-PPD tuberculin (Chiron Italia, Milan, Italy). The size of induration was evaluated after 48-72 hours (an induration  $\geq 10$  mm was classified as positive). Peripheral blood was withdrawn before starting any therapy and under a written informed consent. Healthy controls were selected among HIV-seronegative individuals with no history of TB exposure, no infection and with negative reaction to the TST. Healthy controls were tested for the presence of Ca-Ag responses.

### Ethics Statement.

Written informed consent or consultee approval to enroll was secured for all study participants (patients and healthy donors). This study was approved by the Ethical Committee of the San Raffaele Scientific Institute, the UK National Research Ethics Service (NRES) (reference: 13/LO/1270) and the BSMS Research Governance and Ethics Committee (reference: 13/182/LLE) and carried out in accordance with the approved guidelines. All data were anonymized.

### CMV serology.

Healthy donors were screened for the presence of CMV-specific antibody in serum. CMV IgG serology (Architect CMV IgG, Abbot, Maidenhead, UK) was performed at the Brighton and Sussex University Hospital Trust (BSUHT) virology laboratory. CMV-seropositive and seronegative individuals are referred to as CMV<sup>+</sup> and CMV<sup>-</sup>, respectively.

### **Human samples and T cell cultures.**

Patients and healthy donors used for MTB- and Candida-specific responses were part of a previously published cohort[31]. Donors (male:female, 10:9) used for CMV and SEB-specific responses were 68±17 years old. PBMCs were isolated by blood centrifugation over Ficoll-Hypaque (Sigma/Merck, Darmstadt, Germany) density gradient. Cultures were derived either from freshly-isolated (CMV/SEB responses) or cryopreserved (90% fetal calf serum, FCS, and 10% DMSO; MTB/Ca-Ag responses) PBMCs. Equal numbers of viable (0.1% Trypan Blue-negative) cells were resuspended in complete media (RPMI containing penicillin, streptomycin, glutamine and 10% FCS –all from ThermoFisherScientific- or autologous serum) with or without human recombinant IL-7 (50 ng/ml, unless specified otherwise in individual figures; R&D Systems/Biotechne, Minneapolis, USA) for 7 days. Where indicated, cells were stained with the fluorescent dye 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE, ThermoFisherScientific; 1 µM) as by manufacturer instructions, using autologous serum or FCS to quench the labelling. Where indicated, cells were first cultured in complete medium with or without bacterial *Staphylococcus aureus* enterotoxin B (SEB, 1µg/ml, Sigma/Merck). After 5 days, cells were harvested, washed and counted. Equal number of viable cells were finally seeded in culture with or without IL-7, for additional 7 days (day 12). CSA (0.5 µg/ml, Calbiochem/Merck) or anti-LFA-1 blocking antibody (5 µg/ml, a gift from Prof Ruggero Pardi, University Vita-Salute San Raffaele, Milan) were added to the cultures.

### **MTP- and Ca-Ag-specific ELISPOT assay.**

The ELISPOT for IFN $\gamma$  detection was performed as before[31]. Briefly, equal numbers of viable cells ( $5 \times 10^4$  cells/well) were seeded in duplicate in 96-well plates (MAIPS4510; Millipore/Merck), pre-coated with anti-IFN $\gamma$  capture mAb (B-B1; Diaclone, Besançon, France), together with autologous irradiated PBMCs ( $5 \times 10^4$  cells/well) and *Mycobacterium tuberculosis* peptides (MTPs, a pool of six synthetic peptides; 2 µg/ml per peptide; Primm, Milan, Italy; Ca-Ag,



25 µg/ml; Bio-Rad, Hercules, USA) for 18hrs at 37°C in 5% CO<sub>2</sub> atmosphere. Biotinylated anti-IFN $\gamma$  detection mAb (B-G1; Diaclone) was added (4h), followed by the streptavidin-alkaline phosphatase conjugate (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany; 1h). After a washing step, the nitroblue tetrazolium-BCIP (5-bromo-4-chloro-3-indolylphosphate; Sigma) chromogenic substrate was added. Individual spot forming cells (SFC) were counted using an automated image analysis system ELISPOT reader (AID-GmbH, Strassberg, Germany). MTPs (20 amino-acids) were derived from the ESAT-6 and CFP-10 secretory proteins of MTB, purified (>70%) and previously validated[31]. PBMCs in medium alone or stimulated with phytohemagglutinin (PHA-P; Sigma; 5 µg/ml) were used to assess assay background and functionality.

#### **Flow Cytometry analysis and intracellular cytokine staining (ICS).**

Cells were harvested, washed with staining buffer (PBS supplemented with 0.5% FCS and 0.02% NaN<sub>3</sub>) and incubated with monoclonal antibodies directed against surface Ags for 15 minutes at room temperature. The following fluorescence-conjugated monoclonal antibodies (mAbs) were used: anti-CD3-v500, anti-CD8-Allophycocyanine(APC)-H7, anti-CD27-Phycoerythrin(PE), IL-2-Fluorescein-iso-thio-cyanate(FITC), TNF $\alpha$  Alexa700, (all from BD Biosciences, New Jersey, USA), anti-CD4-Peridinin chlorophyll(PerCP), anti-IFN $\gamma$  PE-Cy7(Cyanine-7), anti-CD154 Pacific-Blue (BioLegend, Cambridge, UK), anti-CD45RA ECD (Beckman Coulter, UK) and Yellow live-dead stain (ThermoFisherScientific). Thereafter, cells were washed in staining buffer prior to acquisition.

ICS was used to measure MTP-specific cytokine release at single-cell level. Equal numbers of CFSE-labeled cytokine-cultured cells ( $0.6 \times 10^6$ ) were stimulated (6hrs) with unpulsed (nil) or MTP-pulsed (4 µg/ml) autologous irradiated (5000 rad) PBMCs ( $3 \times 10^6$  cells), in the presence of human anti-CD28-stimulating mAb (2 µg/ml, BD Biosciences). In the last 5hrs of stimulation, Brefeldin A (10 µg/ml, Sigma) was added. To measure CMV-/SEB-specific cytokine release, equal

numbers of cells were stimulated with CMV lysate (2 µg/ml; Advanced Biotechnologies, Eldersburg, USA) or SEB (1 µg/ml) for 2hrs followed by 14hrs in the presence of Brefeldin A. Thereafter, cells were washed, surface-stained as described above, fixed, permeabilized and stained with anti-CD4, anti-IL-2, anti-IFN $\gamma$  and/or anti-TNF $\alpha$  mAbs. Events were acquired on a BD FACSCalibur or LSRII and data were analysed using the FlowJo-v9.x software (TreeStar Inc., Ashland, USA).

### **Statistical analysis.**

GraphPad Prism 7.03 was used for statistical analyses. The D'Agostino and Pearson and the Shapiro-Wilk tests were used to determine normality of data distribution. Only for normally distributed data that passed both tests, means and standard deviation (SD) are shown and paired t-tests are used to compare 2 groups. For not-normally distributed data, non-parametric paired tests (Wilcoxon test) were used to compare 2 groups. For multiple-group comparisons, 2-way ANOVA with Sidak's or Tukey's multiple comparison corrections were used, as indicated in the figures. Levels of significance are: \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.005$ ; and \*\*\*,  $p \leq 0.0005$ , unless specified differently in individual figures.

## Results

### IL-7 enhances recall responses and proliferation of MTB-specific CD4<sup>+</sup> T cells.

To investigate putative effects of IL-7 on pathogen-specific T cells, we first analyzed chronically infected tuberculosis (TB) patients. These were chosen based on clinical history and manifestation of acute MTB infection (clinic and culture confirmed), positive reaction to the tuberculin skin test (TST) and ability of PBMCs to respond to a stimulation with MHC-II-restricted MTB-specific promiscuous peptides (MTPs) in IFN $\gamma$  ELISPOT assays[31]. Patients with detectable (Pt#1 and Pt#2, Fig. 1) or undetectable (Pt#3, Fig.1) MTB-specific IFN $\gamma$  producing T cells were analyzed. Cryopreserved cells were tested in MTP-recall assays immediately after thawing (d0, Fig. 1A-B) or at the end of a culture (d7) in IL-7 compared to complete media (med). Not all patients with detectable MTP-specific T cell responses on fresh PBMCs[31] had a detectable MTP-response after thawing, as seen in the case of Pt#2, compared to Pt#1. Nonetheless, we found that IL-7 selectively enriched cultures for MTP-specific IFN $\gamma$ <sup>+</sup> T cells by 4-10 fold in all cases (Fig. 1B). While MTP-specific T cells could expand also in control cultures (as seen for Pt#1, med), this was best explained by the increase of total CD3<sup>+</sup>CD4<sup>+</sup> T cells in some patients (not shown). Significantly, the frequency of MTP-specific cells increased in IL-7 cultures over the levels found in control cultures, even if the percentage of total CD3<sup>+</sup>CD4<sup>+</sup> T cells remained similar. Of note, IL-7 also allowed us to detect MTP-specific T cells in samples derived from immunosuppressed patients (i.e. anergic, Pt#3). Sensitization with IL-7 significantly increased absolute numbers of MTP-specific T cells in all the TB patients analyzed (Fig. 1C, n=5) compared to non-BCG (*Bacillus Calmette Guérin*) vaccinated healthy donors (non-MTB-infected controls, n=8). To independently confirm the presence of MTP-specific T cells, we additionally performed ICS after MTP stimulation. MTP-pulsed irradiated autologous PBMCs were used as Ag-presenting cells (APCs). As with ELISPOT assays, in ICS we found that the frequency of MTP-specific, IL-2<sup>+</sup>IFN $\gamma$ <sup>+</sup> CD4 T cells increased in IL-7 cultures, compared to freshly thawed PMBCs (d0) and control cultures (med, Supplementary Fig. 1).

Thus, MTB-specific T cell recall responses are enhanced after sensitization with IL-7.

## **IL-7 supports fungal and viral-specific CD4 T cell accumulation in PBMCs from infected individuals.**

We then asked whether exposure to IL-7 would also enable the accumulation of CD4 T cells specific for fungal Ags derived from endemic pathogens, such as *Ca*. Freshly thawed PBMCs derived from individuals with recurrent *Ca*-infections were immediately analyzed for IFN $\gamma$  expression in *Ca*-Ag-recall ELISPOT, in the presence of Ag-pulsed irradiated autologous PBMCs as APCs (d0, Fig. 2), or seeded in culture with or without IL-7 (Fig. 2, d7 and med, respectively), and analyzed after 7 days. As in the case of TB-specific T cells, also *Ca*-Ag-specific, IFN $\gamma$  spots were remarkably increased in IL-7-driven cultures, compared to controls (d0 and med; Fig 2A). In multiple individuals (n=4), *Ca*-Ag specific effectors were significantly increased by ~10-fold over the levels found after thawing (d0) upon IL-7 exposure compared to control medium (Fig. 2B).

As CMV-specific CD4 T cells have been recently shown to significantly expand in elderly individuals chronically infected with the endemic CMV[32, 33], we investigated CMV-specific CD4 T cell responsiveness to IL-7 in cultures derived from CMV-seropositive (CMV<sup>+</sup>) patients. CMV-seronegative (CMV<sup>-</sup>) individuals were used as controls. Freshly derived PBMCs were cultured in the presence of IL-7 or control medium (med) for 7 days, and then CMV-recall responses were tested in ICS, using total CMV lysate as a source of viral Ags (Fig. 3A, top). Again, cells cultured in IL-7 tended to show higher CMV-recall responses compared to controls (Fig. 3A). Of note, these CMV-specific T cells were mostly poly-functional, TNF $\alpha$ <sup>+</sup>IFN $\gamma$ <sup>+</sup> producing cells (Fig. 3A) and remained undetectable in cultures derived from CMV<sup>-</sup> individuals (Supplementary Fig. 2A). As we found that T cells express varying amounts of CD127 and upregulate it in control medium (Supplementary Fig. 2B), as expected[34], we first rested freshly thawed PBMCs for 5 days, and then subjected them to IL-7-driven cultures (Fig. 3B, top). Under these conditions, we found that IL-7 more potently and reproducibly enabled the accumulation of CMV-specific T cell responses from CMV<sup>+</sup> individuals (Fig. 3C, n=10). IFN $\gamma$ <sup>+</sup> cells expressed low levels of CD127, indicative of IL-7-driven receptor down-modulation[34], upon cytokine-driven activation (Fig. 3D), while maintaining

high levels of LFA-1 (not shown) and polyfunctional cytokine expression (Fig. 3B). Thus, in addition to bacterial also fungal and viral-specific responses are enhanced by IL-7 signals.

### **IL-7 promotes responsiveness of *Staphylococcus aureus* specific T cells.**

Bacterial superantigens, such as the *Staphylococcus aureus* enterotoxin B (SEB), can lead to T cell anergy[35] or suppression[36]. We asked whether culturing the cells in IL-7 might also help SEB-specific T cell responses (Fig. 4). PBMCs from healthy donors were rested 5 days in control medium and then left untreated or cultured in IL-7 for 7 days. SEB-recall responses were then tested in ICS (Fig. 4A, top). We found that while SEB-specific T cells could be detected in control PBMC cultures (med), IFN $\gamma$ <sup>+</sup> or TNF $\alpha$ <sup>+</sup> and TNF $\alpha$ <sup>+</sup>IFN $\gamma$ <sup>+</sup> cells were all enriched for in IL-7-driven cultures from many individuals, significantly (Fig. 4A-B, n=17). As seen for CMV-specific T cells (Fig. 3D), cytokine<sup>+</sup> SEB-specific cells expressed lower levels of CD127 (Fig. 4C) while maintaining high levels of LFA-1 (not shown).

We also tested whether IL-7 is capable of supporting SEB-responsiveness even after Ag-driven expansion, in vitro (Fig. 4D). We found that IL-7 significantly enhanced responses after SEB-restimulation (Fig. 4D). Thus, culturing T cells in IL-7 either before or after Ag-recall allows for a higher frequency of Ag-specific cells to be identified.

### **IL-7 drives cyclosporine A-sensitive TCM cell division.**

In a previous mouse study, we found that IL-7 favored the in vitro expansion of tumor-Ag-experienced T cells, by promoting their proliferation[29]. Thus, we asked whether IL-7-driven accumulation of human, pathogen-specific T cells also requires cell division. To this aim, we set up CFSE-labeled PBMC cultures (Fig. 5A-B). We found that while a small fraction of CD4 T cells derived from healthy donors (~1-10%) underwent several rounds of “spontaneous” (i.e., in the absence of any introduced stimulation) cell-division in complete media (Fig. 5A, top panel), dividing cells were dramatically increased upon IL-7 addition (Fig. 5A, bottom panel). These cells expressed high levels of Bcl-2 (Supplementary Fig. 3), in agreement with the pro-survival role of the cytokine[20]. Importantly, IL-7 favored the expansion of two cell populations, distinguishable for

their proliferation potential (i.e., either fast or slow; F and S respectively in the figures) and with distinct mechanistic requirements. Indeed, while both populations were best detected in high-density cell cultures (Supplementary Fig. 4), they proved differentially sensitive to cyclosporine A (CSA). Fast- but not slow-dividing cells were sensitive to CSA inhibition (Fig. 5B). Additionally, fast-dividing cells were sensitive to anti-LFA-1 mAb blockade (Supplementary Fig. 5), suggesting that cell-to-cell contacts are also required for their proliferation. Of note, IL-7 elicited similar effects in autologous sera and FCS (Supplementary Fig. 4). We reckoned that, while slow-dividing cells represent cells undergoing homeostatic expansion, known to occur via CSA-insensitive mechanisms[19] (Fig. 5B, right panel in top row), fast-dividing cells may represent a distinct population of Ag-experienced memory cells proliferating via a CSA-sensitive mechanism (Fig. 5B, middle-right panel in top row). Flow cytometry analysis indicated that while both  $T_{CM}$  ( $CD45RA^-$ ,  $CD62L^+$ ) and  $T_{EM}$  ( $CD45RA^-$ ,  $CD62L^-$ ) cells accumulated in response to IL-7 (Fig. 5B middle and bottom panel), only fast-dividing  $T_{CM}$  ( $CD45RA^-$ ,  $CD62L^+$ ) cells appeared sensitive to CSA (Fig. 5B, bottom panel). Interestingly, the IL-7-driven accumulation of CMV (Fig. 5C, top), SEB (Fig. 5C, bottom and right, n=15) and MTB-specific (Supplementary Fig. 6) polyfunctional memory T cells was also completely dependent upon CSA-sensitive signaling. Together our data indicate that IL-7-driven cultures might recapitulate the events accounting for the maintenance of Ag-experienced memory T cell subsets in vivo[15], and improve their identification and selection in vitro.

## Discussion

In this study, we demonstrate that IL-7 supports the in-vitro expansion of human pathogen-specific T cells, favoring and, in some instances, enabling their enumeration and characterization. IL-7 sensitive pathogen-specific effectors included cells specific for endemically diffused pathogens (e.g., *Staphylococcus aureus*, *Candida albicans*, CMV and MTB, in parts of the world), infecting humans recurrently during the lifetime, persisting (MTB) and/or reactivating in the body (CMV). Thus, our data will help the study and isolation of such pathogen-specific T cells and others relevant to various clinical settings, including harmful infections, especially in the case of immune-suppressed (HIV, transplantation, aged etc.) or chronically infected individuals, in which pathogen-specific T cells might be low in frequency and/or hypo-responsive.

Previous studies showed that IL-7 can support immune-cell reconstitution in lymphopenic conditions[22-25], restore sepsis-induced lymphocyte dysfunctions([37, 38]), enhance effector function of autoreactive T cells[8-13], and expand tumor-reactive T cells[29, 30]. We then asked whether also pathogen-reactive T cells benefited from IL-7 exposure. Our results show that IL-7 promoted the selective expansion of a fraction of memory CD4<sup>+</sup> T cells containing pathogen-specific cells, best observed in high cell-density cultures. Although IL-7 significantly enhanced overall cell recovery (by ~1.5-fold), cytokine-producing CD4 T cells were not enriched at polyclonal level (as detected by PMA and Ionomycin stimulation, Supplementary Fig. 7) unlike seen for pathogen-specific cells. These cells showed a fast rate of proliferation, sensitive to CSA and LFA-1 inhibition, both in autologous sera and FCS. Thus, neither homeostatic cell division[19, 39-42], known to be CSA insensitive[19, 42], nor food-related, bovine Ags appear to account for the accumulation of fast-dividing cells. Rather, our results suggest the existence of a cell-associated ligand capable of synergizing with IL-7 signals to promote the proliferation and responsiveness of pathogen-specific T cells. We speculate that self-Ag/TCR-initiated signals may play a role in the IL-7 cultures.



Although further research is needed to identify such signals in IL-7 cultures, our data support a role for IL-7 in T cell-driven immunopathology in chronic and persistent infections, co-infections or autoimmunity. Accordingly, IL-7 is expressed in inflamed tissues of patients with (rheumatic) autoimmune diseases, where it can be produced by several cell types[43] (including macrophages, dendritic cells, and fibroblasts) and favor pathogenic Th1 and Th17-associated cytokine secretion. Furthermore, dysregulated IL-7 expression or activation of CD127 were found in patients with autoimmune conditions[8-13], suggesting that IL-7 supports the function of pathogenic effector cells in autoimmunity. In agreement, blocking the IL-7R in experimental animal models ameliorated autoimmune disease manifestations[44]. Thus, together with available data, our results suggest that IL-7 might awaken auto-reactive T cells, or pathogen-specific effector T cells with cross-reactivity to self-antigen, hence contributing to autoimmunity. However, this might not equally apply to patients with immune dysfunctions due to sepsis or chronic HIV/HCV infection. Indeed, IL-7 administration mainly in HIV-infected or immunosuppressed patients was generally well-tolerated[22, 23, 25, 27, 28, 45], with a single report of a patient developing systemic lupus erythematosus after 3 doses of IL-7[45]. We speculate that the risk of developing autoimmunity after IL-7 treatment may vary dependent on individual clinical history, genetic predisposition and the administration regimen. Further studies are needed to define the long-term consequences of IL-7 administration.

Interestingly, among memory cells, polyfunctional T cells (double positive for IFN $\gamma$ <sup>+</sup> and IL-2<sup>+</sup> or TNF $\alpha$ <sup>+</sup>) were mostly enriched for by IL-7. Such cells were detected in subjects with chronic viral (including CMV[32]) infections and previously referred to as intermediate polyfunctional memory cells[46]. It is possible that IL-7 favors differentiation of these cells in vitro (and possibly also in vivo), starting from IFN $\gamma$ -producing cells. With respect to maintaining polyfunctional T cells, IL-7 appears superior to the cognate Ag by favoring (central) memory cell survival[15-17], rather than terminal differentiation, activation induced cell-death (AICD) and/or exhaustion[29]. Thus, IL-7 may be useful for the expansion of human polyclonal and polyfunctional pathogen-specific CD4 (and to a lower extent, CD3<sup>+</sup> CD4<sup>-</sup> or CD8<sup>+</sup>, Supplementary Fig. 8A-E) T cells hard to be identified,



even in the case of relatively well-studied pathogens (such as CMV[47, 48]). Accordingly, exposing cells to IL-7 enabled better enumeration of in vivo-primed CMV/SEB-specific cells, although these trends were less apparent in CD8<sup>+</sup> T cells, which did not undergo fast-proliferation to the extent of CD4 T cells (Supplementary Fig. 8F). This was also the case when T cells were Ag-re-stimulated in vitro prior to the IL-7 culture, opening the possibility that IL-7 (with or without Ag) might be superior to Ag alone in expanding T cells derived from in vivo-primed individuals. Our data also support the hypothesis that IL-7 sustains the preferential accumulation of polyfunctional T cell subsets within the repertoire of certain individuals, including perhaps the inflated responses of CD4 T cells detected in old CMV<sup>+</sup> individuals[32, 33]. Future studies are needed to address this possibility.

Previously, we suggested that among other CD132-cytokines, IL-7 played non-redundant roles and outperformed IL-2 in driving Ag-experienced T cell accumulation and mediating the expansion of less differentiated cells useful for gene therapy[26, 30]. We now provide evidence supporting the use of IL-7 to reveal and expand in vivo-primed pathogen-specific lymphocytes of clinical relevance, either as biomarkers of viral infection and disease activity[49], or as therapeutic tools. This may be relevant for the treatment of chronic infectious diseases and cancer, since adoptive immunotherapy with less differentiated T cells is preferable over the transfer of terminally differentiated effectors[50].

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## Figure legends

**Figure 1. IL-7 enhances *Mycobacterium tuberculosis*-specific T cell responses.** **A.** Cryopreserved PBMCs from TB patients were analyzed for IFN- $\gamma$  release by MTP-specific ELISPOT assay at the time of thawing (d0) and after a 7-day culture in the absence (med, dashed line) or in the presence of human recombinant IL-7 (IL-7, solid line), in parallel to unstimulated controls (nil). For each patient, 2 duplicate vials were sequentially thawed, staggered in time (dashdotted lines): the first vial was used to set up the cultures at d0 (left dashdotted arrow); the second vial was thawed 7 days later (right dashdotted arrow) in order to provide for: (i) ex vivo control cells to be analyzed in parallel to cultured cells; and (ii) autologous feeder cells for the restimulation assays (refer to Materials and Methods for details). **B.** Background IFN $\gamma$  release was measured for any condition in Ag-unpulsed control wells (nil), as representatively depicted for Pt. #1 (top row). IFN $\gamma$ -spots after re-stimulation with MTP promiscuous peptides were detected in three TB patients (Pt. #1, #2 and #3) after thawing (d0) and/or a 7-day culture (d7) in IL-7 (IL-7) or control medium (med). **C.** The effect of the IL-7 treatment (d0 versus d7, IL-7) on MTP-specific IFN $\gamma$ -spot accumulation in PBMCs derived from healthy donors (open circles, n=8) and MTB-infected patients (open triangles, n=5) was analyzed by the ELISPOT assay in multiple individuals. Statistical significances were evaluated using a 2-way ANOVA with Sidak's multiple comparison correction to evaluate the effect of IL-7 treatment in the two different groups during time, and the diagnostic power of the discrimination of the two groups before and after IL-7 treatment, as indicated: \*, p $\leq$ 0.05; \*\*, p $\leq$ 0.005.

**Figure 2. IL-7 supports the expansion and effector function of *Candida albicans*-specific T cells.** **A.** After thawing, PBMCs from a donor with recurrent *Candida albicans* (Ca) infection were analyzed for the release of the effector cytokine, IFN $\gamma$  using a Ca-Ag-specific ELISPOT assay at the time of thawing (d0) and after a 7-day culture in the absence (d7, med) or in the presence of human recombinant IL-7 (d7, IL-7). Background IFN $\gamma$  release was measured in Ag-unpulsed

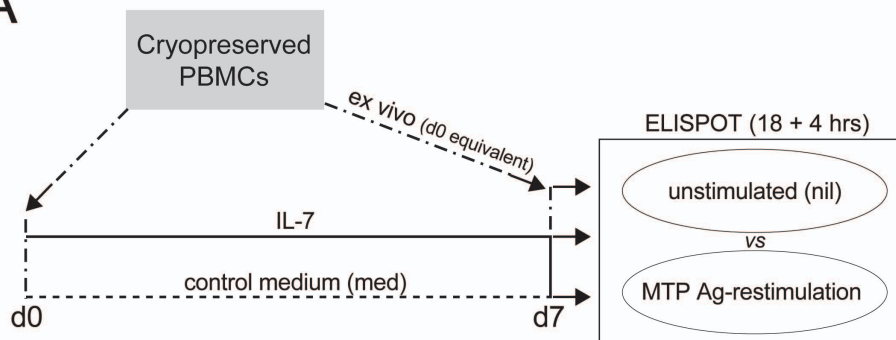
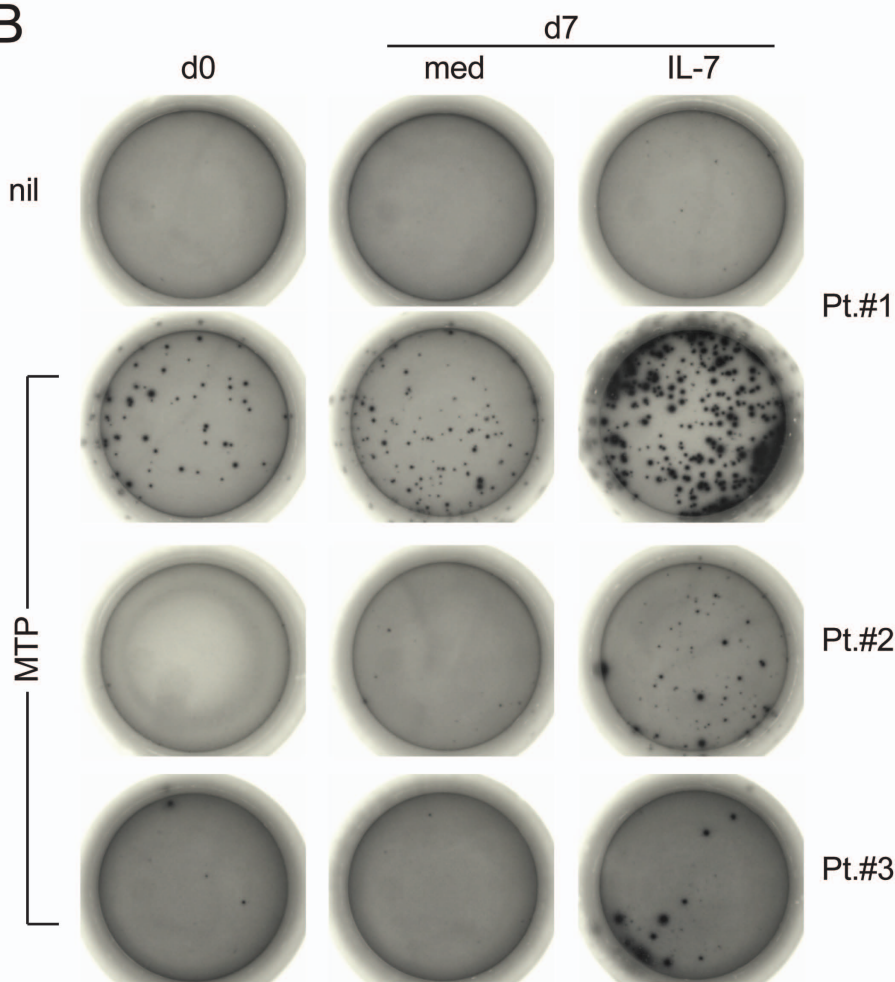
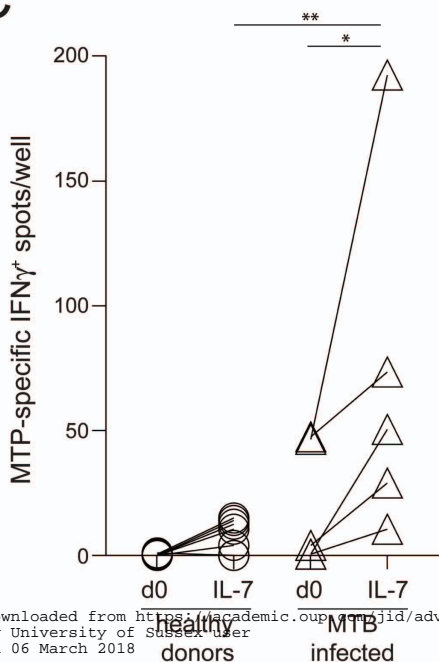


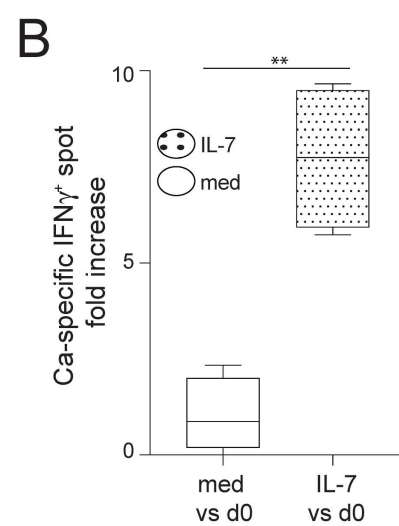
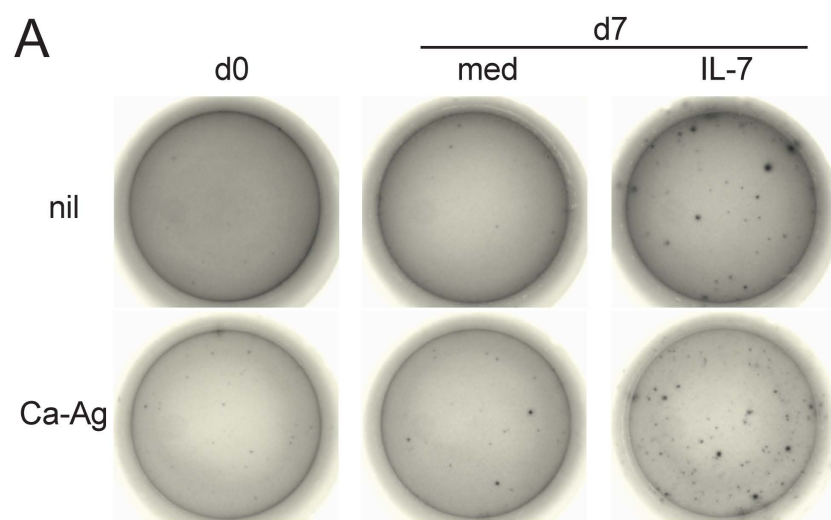
control wells (nil, upper wells) in the presence of irradiated autologous feeder cells. **B.** The range and average fold-increase of Ca-Ag-specific IFN $\gamma$ -spots in control medium (med vs d0, white bar) and IL7 (IL7 vs d0, dotted bar) cultures over the levels found after thawing (d0) is shown. Statistically significant accumulation of Ca-Ag-specific IFN $\gamma$ -spots was evaluated in multiple donors (n=4) using a paired t-test. \*p $\leq$ 0.05; \*\*, p $\leq$ 0.005; \*\*\*, p $\leq$ 0.0005.

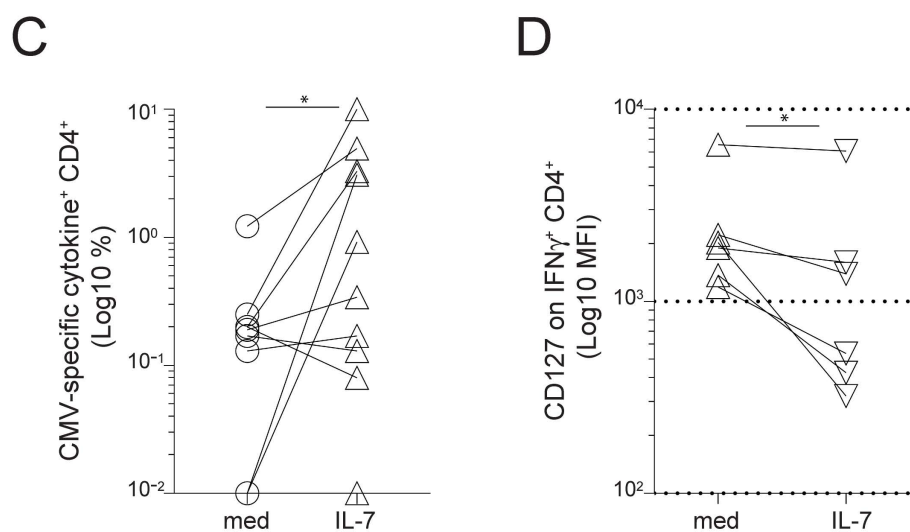
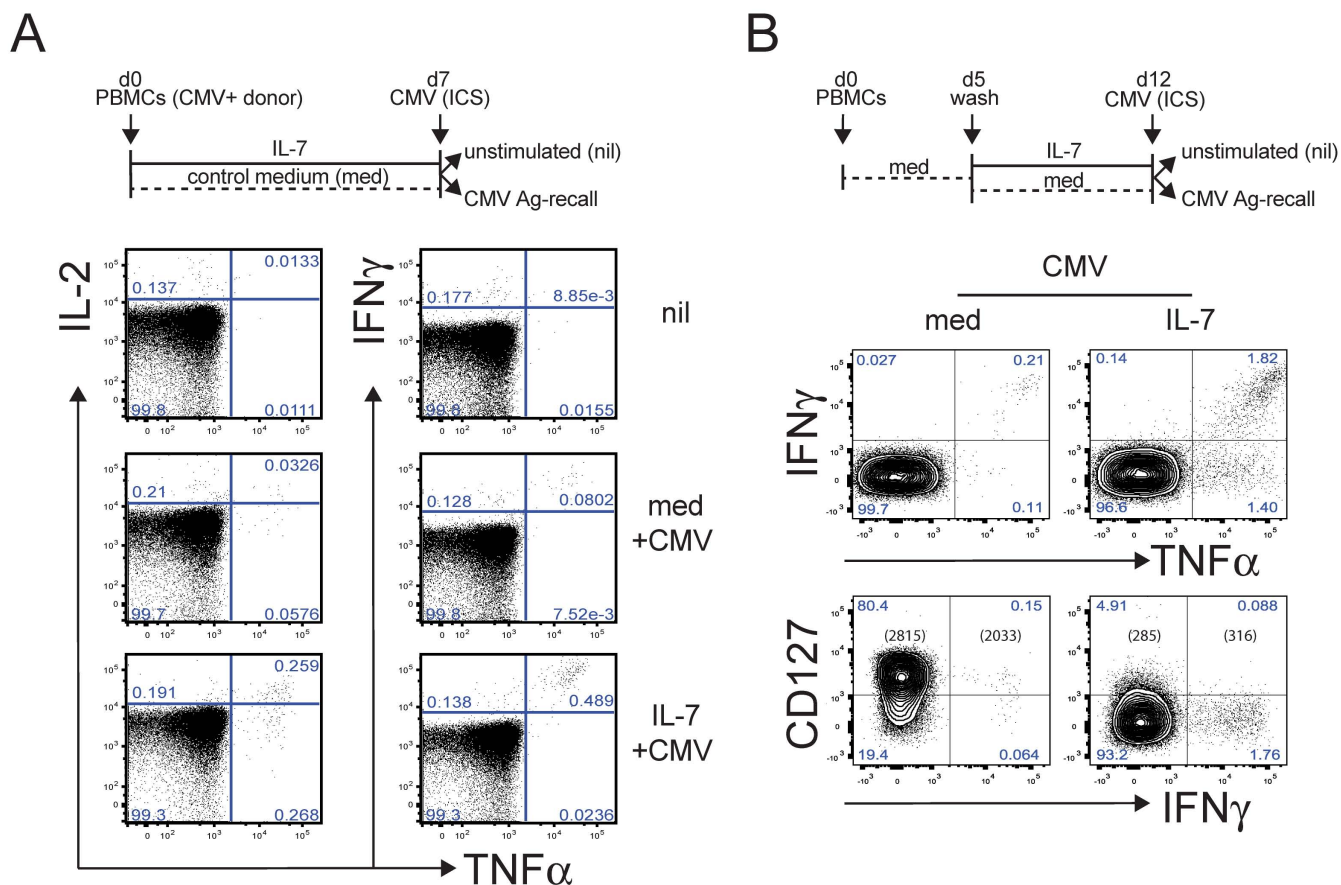
**Figure 3. IL-7 supports the accumulation of antiviral CMV-specific T cells.** **A.** Freshly-derived PBMCs from CMV<sup>+</sup> donors were analyzed for inflammatory cytokine release after CMV-lysate ICS assay after a 7-day culture in the absence (d7, med, dashed black line) or in the presence of human recombinant IL-7 (d7, IL-7, black line). Left and right dot plots show the levels of IL-2 and TNF $\alpha$  or IFN- $\gamma$ , and TNF $\alpha$  in gated CD4 T cells, respectively. Background levels of cytokine secretion were typically measured in unstimulated controls (nil). **B.** Freshly isolated PBMCs from CMV<sup>+</sup> donors were rested for 5 days in plain medium (dashed black line) prior to a 7-day culture in the absence (d12, med, dashed black line) or in the presence of human recombinant IL-7 (d12, IL-7, black line). At day 12 (d12), CD4<sup>+</sup> T cells were analyzed for IFN- $\gamma$  and TNF $\alpha$  release (top row) alongside expression of CD127 (bottom row), after CMV-lysate ICS assay. CMV-specific IFN- $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells show high CD127 expression in the resting cultures whilst they downregulated CD127 expression upon exposure to IL-7. Background levels of cytokine secretion were typically measured in ICS unstimulated (nil) controls. A representative of >10 independent experiments is shown. **C.** After subtraction of individual background levels of IFN- $\gamma$ <sup>+</sup> and TNF $\alpha$ <sup>+</sup> and IFN- $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup> CD4 T cells detected in unstimulated controls (nil), the percentage of CMV-specific cytokine<sup>+</sup> (IFN- $\gamma$ <sup>+</sup> and TNF $\alpha$ <sup>+</sup>) CD4 T cells was evaluated in 10 independent CMV<sup>+</sup> donors in IL-7 (IL-7, open triangles) compared to control medium (med, open circles) cultures. The graph shows a statistically significant (paired Wilcoxon test, \*p=0.03) increase of the frequency (Log10) of CMV-specific CD4 T cells after IL-7 culture. **D.** CD127 expression (Log10 MFI, mean fluorescence intensity) is significantly downregulated in CMV-specific IFN- $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells exposed to IL-7 (IL-7) compared to control medium (med) at d12 in 6 biological independent replicates (paired Wilcoxon test, \*p=0.03).

**Figure 4. IL-7 supports superantigen-specific responses.** **A.** Freshly isolated PBMCs were rested for 5 days in plain medium (dashed black line) then incubated for one week in IL-7 or control medium (respectively: IL-7, black line and, med, dashed black line). At d12, cells were stimulated with SEB during an ICS assay to test Ag-specific  $\text{TNF}\alpha$  and  $\text{IFN-}\gamma$  release, compared to unstimulated controls (nil). Dot plots show that the frequency of SEB-specific  $\text{CD4}^+$  T cells producing  $\text{TNF}\alpha$  and/or  $\text{IFN-}\gamma$  increased upon exposure to IL-7 in one representative donor. **B.** After subtraction of individual background levels of  $\text{IFN-}\gamma^+$  and  $\text{TNF}\alpha^+$  and  $\text{IFN-}\gamma^+\text{TNF}\alpha^+$   $\text{CD4}^+$  T cells detected in unstimulated controls (nil), the percentage of SEB-specific cytokine<sup>+</sup> ( $\text{IFN-}\gamma^+$ ,  $\text{TNF}\alpha^+$ ,  $\text{IFN-}\gamma^+\text{TNF}\alpha^+$  and total)  $\text{CD4}^+$  T cells was evaluated in 17 independent donors in IL-7 (IL-7, open triangles) compared to control medium (med, open circles) cultures. The graphs show statistically significant increase of the frequency (Log10) of SEB-specific cytokine<sup>+</sup>  $\text{CD4}^+$  T cells after IL-7 culture. All tests are paired Wilcoxon tests with the exception of the single  $\text{TNF}\alpha^+$  analysis (paired t-test): \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.005$ ; \*\*\*,  $p \leq 0.0005$ . **C.** At d12, cells cultured as in (A) were tested for CD127 expression in parallel to cytokine release in SEB-specific ICS assay. CD127 expression (Log10 MFI, mean fluorescence intensity) is significantly decreased in SEB-specific  $\text{IFN-}\gamma^+$   $\text{CD4}^+$  T cells exposed to IL-7 (IL-7, downward triangles) compared to control medium (med, upward triangles) in 12 independent biological replicates (paired Wilcoxon test, \*\*\* $p=0.0005$ ). **D.** Freshly isolated PBMCs (d0) were stimulated with the SEB superantigen (dotted black line) for 5 days (d0-5). At d5, cells were washed and incubated for one week in IL-7 (IL-7, black line; d5-12) or control medium (med, dashed black line; d5-12). At d12, cells were stimulated with SEB (overnight) to test Ag-specific  $\text{TNF}\alpha$  and  $\text{IFN-}\gamma$  release, by ICS. Dot plots show that the frequency of SEB-specific  $\text{CD4}^+$  T cells producing  $\text{TNF}\alpha$  and/or  $\text{IFN-}\gamma$  increased upon exposure to IL-7 (IL-7 d5-12, upper rows). The graph on the right shows data from the same cultures derived from 6 independent biological repeats. Statistically significant accumulation of SEB-specific, cytokine<sup>+</sup>  $\text{CD4}^+$  T cells was evaluated using a Wilcoxon matched-pairs signed ranked test ( $p=0.03$ ).

**Figure 5. Sensitization by IL-7 promotes pathogen specific CD4<sup>+</sup> T cells proliferation in CSA-sensitive manner.** CFSE-labeled PBMCs (A-C) from healthy donors (A-B) or TB patients (C) were cultured for 7 days in the absence (med) or in the presence of human recombinant IL-7 (IL-7). **A.** Proliferation of viable CD4 T cells in high density cultures ( $5 \times 10^6$  cells/ml) was determined by flow cytometry after staining with anti-CD4 mAb and TO-PRO-3 (an intercalant agent of DNA entering necrotic and apoptotic cells). Dot plots depict the relative CFSE content within the same number of viable, TO-PRO-3<sup>-</sup> CD4<sup>+</sup> T cells. **B.** PBMCs cultured ( $4 \times 10^6$  cells/ml) in the presence of IL-7 for 7 days in the absence or in the presence of CSA. At d7, cells were stained with anti-CD4, antiCD45RA and anti-CD62L mAb and the relative CFSE content was analyzed by flow cytometry in total CD4 T cells (top row). Fast- (F), Slow- (S), and non-dividing cells were determined based on CSA inhibition, as indicated. The percentage of naïve (CD45RA<sup>+</sup> CD62L<sup>hi</sup>), central memory (CD45RA<sup>-</sup> CD62L<sup>hi</sup>), and effector memory (CD45RA<sup>-</sup> CD62L<sup>lo</sup>) cells were then investigated in slow-compared to fast-proliferating CD4 T cells. **C.** Freshly isolated PBMCs were rested for 5 days then incubated for one week in IL-7 or IL-7 plus CSA (IL-7+CSA). At d12, cells were stimulated with SEB during an ICS assay to test Ag-specific TNF $\alpha$  and IFN- $\gamma$  release, compared to unstimulated controls (refer to Figs. 3-4). Left dot plots show that the IL-7-driven accumulation of CMV- (top) and SEB-(bottom)-specific CD4<sup>+</sup> T cells producing TNF $\alpha$  and/or IFN- $\gamma$  is reversed in the presence of CSA, by d12. After subtraction of individual background levels of IFN- $\gamma$ <sup>+</sup>, TNF $\alpha$ <sup>+</sup> and IFN- $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup> CD4 T cells detected in unstimulated controls (nil), the percentage of SEB-specific cytokine<sup>+</sup> (IFN- $\gamma$ <sup>+</sup>, TNF $\alpha$ <sup>+</sup>, IFN- $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup> and total) CD4 T cells was evaluated in 15 independent biological repeats in IL-7 (open circles) compared to IL-7+CSA cultures (open triangles). The right graphs show statistically significant decrease in the frequency (Log10) of SEB-specific cytokine<sup>+</sup> (except for IFN- $\gamma$ <sup>+</sup>) CD4 T cells in the presence of CSA at d12. All tests are paired Wilcoxon tests. \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.005$ ; \*\*\*,  $p \leq 0.0005$

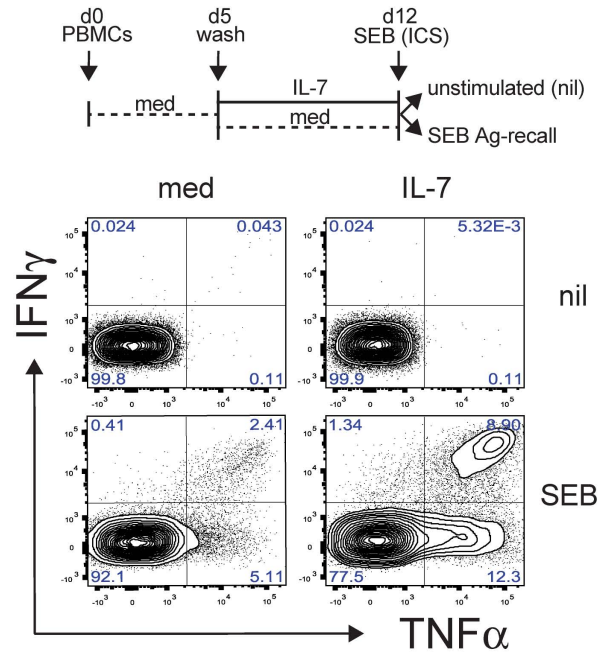
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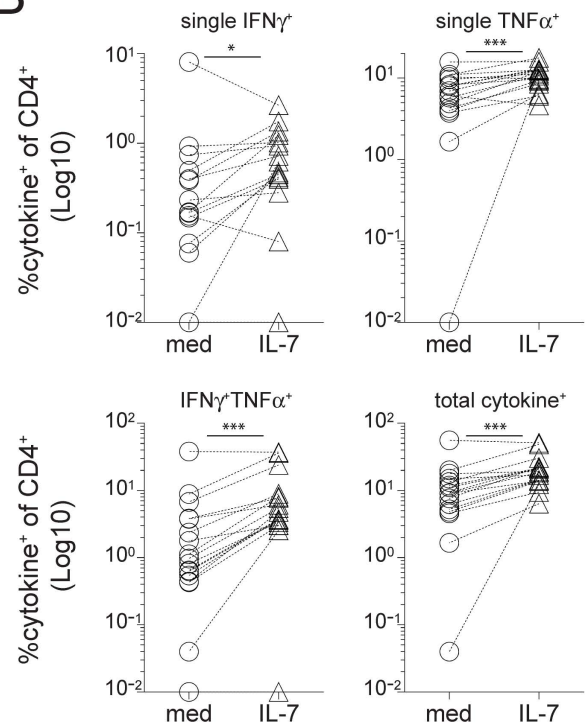




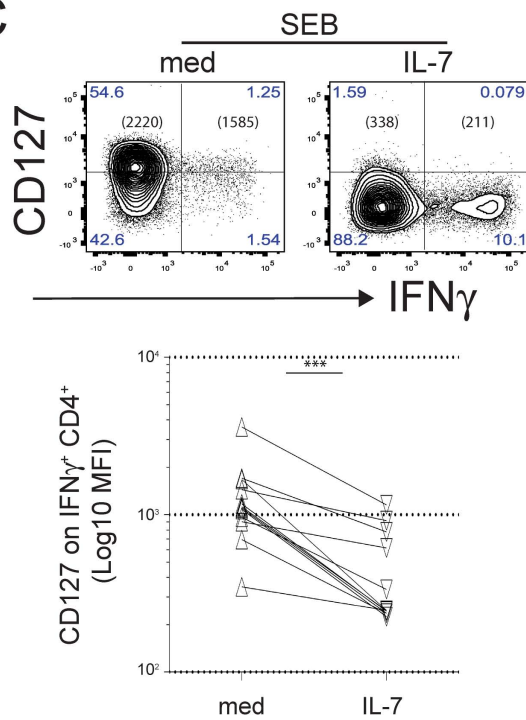
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